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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<p>(21) International Application Number: PCT/US89/02363</p> <p>(22) International Filing Date: 31 May 1989 (31.05.89)</p> <p>(30) Priority data:</p> <table border="0"><tr><td>200,930</td><td>1 June 1988 (01.06.88)</td><td>US</td></tr><tr><td>306,798</td><td>2 February 1989 (02.02.89)</td><td>US</td></tr></table> <p>(71) Applicant: BIOGEN, INC. [US/US]; 14 Cambridge Center, Cambridge, MA 02142 (US).</p> <p>(72) Inventors: RAMACHANDRAN, Kuzhalmannam, L. ; 24 Otis Street, Cambridge, MA 01760 (US). CATE, Richard, L. ; 168 Bayshore Drive, Plymouth, MA 02360 (US).</p> <p>(74) Agents: HALEY, James, F., Jr. et al.; 875 Third Avenue, New York, NY 10022-6250 (US).</p>		200,930	1 June 1988 (01.06.88)	US	306,798	2 February 1989 (02.02.89)	US	<p>(81) Designated States: AT (European patent), AU, BE (European patent), CH (European patent), DE (European patent), FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent).</p> <p><b>Published</b> <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i></p>
200,930	1 June 1988 (01.06.88)	US						
306,798	2 February 1989 (02.02.89)	US						
<p>(54) Title: LABELED NUCLEIC ACID PROBE</p> <p>(57) Abstract</p> <p>A polynucleotide probe with a label bearing a plurality of signalling moieties. The label is attached to the probe by the reaction of an amino and sulfhydryl reactive hetero bifunctional reagent with the probe and label, the reaction resulting in the oxidation of a sulfhydryl group of the label. The label may be attached to the 5' terminus of the probe, or to modified bases of the probe. Probes constructed according to the invention are useful in detecting target sequences in genomic DNA.</p>								

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LABELED NUCLEIC ACID PROBETECHNICAL FIELD OF THE INVENTION

5 This invention relates to polynucleotide probes for detecting target nucleic acid sequences in an analyte. More particularly, this invention relates to labeled polynucleotide probes in which a polypeptide or other organic molecule serves to label the probe and to make it detectable. As will be  
10 appreciated, the labeled probes of this invention are useful in many laboratory, industrial and medical applications wherein detection of a target base sequence in a nucleic acid is desired.

BACKGROUND OF THE INVENTION

15 In this description, the following terms are employed:

Analyte - A substance or substances, either alone or in admixtures, whose presence is to be detected and, if desired, quantitated. The analyte  
20 may be a DNA or RNA molecule of small or high molecular weight, a molecular complex including those molecules, or a biological system containing nucleic acids, such as a virus, a cell, or a group of cells. Among the common analytes are nucleic acids (DNA and  
25 RNA) or segments thereof, either single- or double-stranded, viruses, bacteria, cells in culture, and the like. Also included are fungi, algae, other

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microorganisms, as well as animals (e.g., vertebrates) and plants, their cells, tissues and fluids.

Bridging Moiety - That moiety which on covalent attachment or non-covalent binding to the label of a polynucleotide sequence acts as a connection between the label and a signalling moiety.

Genomic DNA - An analyte comprising the DNA of an organism. Typically, the analyte will be purified nuclear DNA, and may, although not necessarily, include all nucleotide sequences present in the organism's DNA.

Label - That moiety attached to the probe by a linkage group which as is, which after covalent attachment of a signalling moiety or a combination of bridging moiety and signalling moiety to it, or which after non-covalent binding of a signalling moiety or a combination of bridging moiety and signalling moiety to it, gives rise to a signal which is detectable, and in some cases, quantifiable.

Linkage Group - That moiety which serves to link or attach a label to the probe. The linkage group serves to hold the label away from the probe, so as to prevent interference with binding between the probe and target.

Probe - A polynucleotide sequence which is complementary to a target polynucleotide sequence in the analyte.

Signal - That characteristic of a label or signalling moiety that permits it to be detected.

Signalling Moiety - That moiety which on covalent attachment or non-covalent binding to the probe, label or to a bridging moiety attached or bound to the probe or label provides a signal for detection of the label and the moiety to which the label is attached.

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Target - The specific sequence of bases in a nucleic acid present in an analyte whose presence is to be detected.

The analysis and detection of minute quantities of substances in biological and non-biological samples has become a routine practice in clinical and analytical laboratories. These detection techniques can be divided into two major classes: (1) those based on ligand-receptor interactions (e.g., immunoassay-based techniques) and (2) those based on nucleic acid hybridization (polynucleotide sequence-based techniques).

Immunoassay-based techniques are characterized by a sequence of steps comprising the non-covalent binding of an antibody and an antigen complementary to it. See, for example, T. Chard, An Introduction To Radioimmunoassay And Related Techniques (1978).

Polynucleotide sequence-based detection techniques are characterized by a sequence of steps comprising the non-covalent binding of a labelled polynucleotide sequence or probe to a complementary sequence of the analyte under conditions which permit hybridization of the bases through Watson-Crick pairing, and the detection of that hybridization. [M. Grundstein and D. S. Hogness, "Colony Hybridization: A Method For The Isolation Of Cloned DNAs That Contain A Specific Gene", Proc. Nat. Acad. Sci. USA, 72, pp. 3961-65 (1975); D. T. Kingsbury, "DNA Probes In The Diagnosis Of Genetic And Infectious Diseases", Trends In Biotechnology, 5, pp. 107-11 (1987).]

The non-covalent binding of a labelled sequence or probe to a complementary sequence of an analyte is the primary recognition event of polynucleotide sequence-based detection techniques. This binding event is brought about by a precise

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molecular alignment and interaction of complementary nucleotides of the probe and target. It is energetically favored by the release of non-covalent bonding free energy, e.g., hydrogen bonding, stacking free energy and the like.

In order to employ the non-covalent binding of a probe for the determination of an analyte containing a target sequence, it is necessary to be able to detect binding of the probe to the target. This detection is effected through a signalling step or event. A signalling step or event allows detection in some quantitative or qualitative manner of the occurrence of the primary recognition event.

In general, the use of a separate label moiety to attach a signal moiety to a probe is a desirable way to couple the primary recognition and signal events. First, this technique permits attachment of large bridging and signalling moieties to the probe, while interfering only minimally with the structure of the probe, and therefore with its binding to the target. Second, use of a separate label permits the indirect attachment of many signalling moieties to a single locus within a probe molecule. Attachment of a label bearing many signalling moieties at a single point, rather than attaching signalling moieties to many bases, serves to minimize the likelihood that binding between the probe and target will be disturbed, while providing a larger signal than with a single signalling moiety. An additional improvement is to employ a linkage group as a rigid point of attachment between the label and probe, which will hold the label away from the probe during hybridization.

The primary recognition event and the signalling event of polynucleotide sequence based detection techniques may be coupled either directly, proportionately or inverse proportionately. Thus, in

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such systems as nucleic acid hybridization assays performed with detectable probes, the amount of signal is usually directly proportional to the amount of analyte present. Inversely proportional techniques include, for example, competitive assays, wherein the amount of detected signal decreases with increasing amounts of analyte present in the sample.

Amplification techniques are of great importance when only a small amount of a target is present. For example, the signalling component of the assay may be present in a ratio of 10:1 for each recognition component, thereby providing a 10-fold increase in sensitivity.

A wide variety of signalling events may be employed to detect the occurrence of the primary recognition event. The signalling event chosen depends on the particular signal that characterizes signalling moiety employed. Although the label itself, without further treatment, may be detectable, more often, either the signalling moiety is attached covalently, or bound non-covalently to a label or a combination of signalling and bridging moieties in order to render the primary recognition event detectable.

Although the combination of bridging moiety and signalling moiety, described above, may be constructed before attachment or binding to the label, it may also be sequentially attached or bound to the label. For example, the bridging moiety may be first bound or attached to the label and then the signalling moiety combined with the joined label and bridging moiety. In addition, it should be understood that several bridging moieties and/or signalling moieties may be employed together in any one combination of bridging moiety and signalling moiety.

Examples of the covalent attachment of a signalling moiety or a combination of bridging moiety



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and signalling moiety to a label include chemical modification of the label with signalling moieties. In addition, the primary recognition event may be detected by the non-covalent binding of a signalling moiety or a combination of bridging moiety and signalling moiety that itself can be detected by appropriate means, or the non-covalent binding to the label of a combination of bridging moiety and signalling moiety to provide a signal that may be detected by one of those means. For example, the label could be bound to a bridging moiety, e.g., a lectin, and then bound through the lectin, or bridging moiety, to another moiety that is detectable by appropriate means.

There are a wide variety of signalling moieties and bridging moieties that may be employed for covalent attachment or non-covalent binding to the label of polynucleotide sequences useful as probes in analyte detection systems. All that is required is that the signalling moiety provide a signal that may be detected by appropriate means and that the bridging moiety, if any, be characterized by the ability to attach covalently or to bind non-covalently to the label, and also possess the ability to combine with a signalling moiety.

Signalling moieties may be radioactive or non-radioactive. Radioactive signalling moieties are characterized by one or more radioisotopes of phosphorous, iodine, hydrogen, carbon, cobalt, nickel, and the like. Preferably the radioisotope emits  $\beta$  or  $\gamma$  radiation, and has a long half life. Detection of radioactive signalling moieties is typically accomplished by the stimulation of photon emission from crystalline detectors caused by the radiation, or by the fogging of a photographic emulsion.

Non-radioactive signalling moieties have the advantage that their use does not pose the

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hazards associated with exposure to radiation, and that special disposal techniques after use are not required. [D. T. Kingsbury, (1987), p. 108.] In addition, they are generally more stable, and as a  
5 consequence, cheaper to use. Detection sensitivities of non-radioactive signalling moieties may be as high or higher than those of radioactive signalling moieties.

Among the preferred non-radioactive signalling moieties or combinations of bridging and signalling moieties useful with non-radioactive labels are those based on the biotin/avidin binding system [P.R. Langer et al., "Enzymatic Synthesis of Biotin Labeled Polynucleotides: Novel Nucleic Acid Affinity Probes",  
15 Proc. Nat. Acad. Sci. USA, 78, pp. 6633-37 (1981). R. H. Singer and D. C. Ward, "Actin Gene Expression Visualized In Chicken Muscle Tissue Culture By Using In Situ Hybridization With A Biotinated Nucleotide Analog", Proc. Nat. Acad. Sci USA, 79, pp. 7331-35  
20 (1982)]. For a review of non-radioactive signalling and bridging-signalling systems, see U.S. Patent No. 4,711,955.

Non-radioactively labeled polynucleotides are not more widely used in detection systems because  
25 the attachment of a label which does not interfere with hybridization is expensive and because of difficulties in attaching the signalling moiety to the probe. The chemical reaction conditions that might be useful for modification of a polynucleotide to  
30 add it to a label are often too vigorous to be sufficiently selective for a particular nucleotide. More importantly, chemical labelling of polynucleotide sequences often interferes with the hydrogen bonding necessary for hybridization. For example, dicarbonyl  
35 reagents, such as glyoxal and kethoxal, react with guanine residues, but the glyoxal and kethoxal reacted nucleotides do not hybridize to complementary

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sequences in the analyte because the glyoxal or kethoxal moiety interferes with the hydrogen bonding necessary for hybridization [M. Litt, "Structural Studies on Transfer Ribonucleic Acid. I. Labeling Of Exposed Guanine Sites in Yeast Phenylalanine Transfer Ribonucleic Acid with Kethoxal", Biochemistry, 8, pp. 3249-53 (1969)].

An alternative approach, such as that disclosed in U.S. Patent No. 4,711,955, provides for covalent attachment of a label to individual bases by way of a linkage group. Labeled probes must then be constructed from labeled and unlabeled bases using a polynucleotide complementary to the probe as a template. Thus little specificity as to the location of the label on the probe is possible, and as consequence, steric interference between adjacent labels is possible. In addition, synthesis of the labeled monomeric nucleotides prior to incorporation into the polynucleotides involves expensive chemical processes. The coupling of the labelled monomeric nucleotides into a polynucleotide is also expensive, as the cost of the enzymes used in enzymatic coupling is substantial.

A further deficiency of a labeling technique in which individual nucleotides of a probe sequence are labeled is that the signal intensity is generally low. Because a single signalling moiety is attached to each nucleotide base, in order to achieve a level of signal intensity that is readily detectable, a long probe molecule is required. This reduces the ability of the probe to detect minor mismatches between the probe and analyte, since sufficient hydrogen bonding between other bases will cause the probe to remain bound to the analyte during washing. Detection of single mismatches between base pairs is of critical importance in diagnosing certain inherited diseases such as sickle-cell anemia

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[B. J. Conner et al., "Detection of Sickle Cell  $\beta^S$ -Globin Allele By Hybridization With Synthetic Oligonucleotides, Proc. Nat. Acad. Sci. USA, 80, pp. 278-82 (1983)].

5           A particularly important application of the present invention is in screening an organism's entire genome in order to detect a particular target base sequence. For example, it is desirable to detect the presence of inheritable diseases in  
10 potential human parents in cases where the disease does not manifest itself until after child bearing age has been attained, as in Huntington's chorea, and in diseases where heterozygous individuals are merely carriers, as in sickle cell anemia and Tay  
15 Sachs disease. Genomic screening requires a probe having great sensitivity. Moreover, effective genomic screening requires a short probe capable of detecting a mutation at a single base within the affected gene in order to detect inheritable diseases  
20 resulting from alteration at only a single base, such as sickle cell anemia [Conner et al., 1983]. A probe capable of selectively detecting a target polynucleotide sequence in an analyte of genomic DNA or RNA may be termed a genomic probe. To date no genomic  
25 probes apart from those bearing radioactive signalling moieties and those greater than about 400 base pairs in length have been disclosed which are capable of distinguishing mutant from wild type individuals. As will be demonstrated below, short probes prepared  
30 according to the present invention are capable of doing so.

#### SUMMARY OF THE INVENTION

The present invention solves both the problems associated with use of radiolabeled probes and  
35 with prior art methods of probe labeling by providing

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a polynucleotide probe and a label bearing a plurality of signalling moieties. The label is attached to the probe by the reaction of an amino and sulfhydryl reactive hetero bifunctional reagent with the probe and label, the reaction resulting in the oxidation of a sulfhydryl group of the label. The mode of attachment between the probe and label serves to reduce interference during hybridization between the probe and target polynucleotides. In one embodiment the point of attachment is an amino group at the 5' terminus of the probe. In an alternative embodiment, the point of attachment is an amine group affixed to a modified base within the probe. The present invention provides for amplification of the signal resulting from probe binding by permitting attachment of a plurality of signalling moieties to the label. Another aspect of the invention provides a method for detection of alterations in the sequence of nucleotides in genomic DNA. The method employs the labeled nucleic acid probes of this invention, and is sensitive enough to detect alterations of a single nucleotide within a gene.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic outline of the chemical reactions employed in activating the 5' -OH group of the polynucleotide probe.

Figure 2 is a schematic outline of the chemical reactions employed in attaching the label to the activated polynucleotide probe.

#### DETAILED DESCRIPTION OF THE INVENTION

According to the present invention, a nucleic acid probe molecule with a nucleotide sequence complementary to that of a target sequence in the analyte is labeled by covalently attaching an organic molecule comprising a reactive sulfhydryl group to

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the 5' terminus of the probe. In a preferred embodiment, the label is a polypeptide strand with a cysteine residue at its amino terminus. Alternatively, the label may be any molecule with reactive amino groups and at least one sulfhydryl group. The polypeptide strand includes, preferably, a plurality of residues bearing reactive carboxyl and/or amino groups. As examples, lysine and ornithine possess reactive amino groups, while aspartic acid and glutamic acid possess reactive carboxyl groups. In the preferred embodiment, bridging moieties are attached to the polypeptide after the polypeptide is attached to the probe. Preferred bridging moieties include biotin and iminobiotin. After the labeled probe has hybridized to an analyte, these labels may be detected by exposing them to a signal moiety, or to a second bridging moiety with a signalling moiety attached thereto. Preferably avidin or streptavidin are the second bridging moieties to which a signal moiety has been attached.

In an alternative embodiment, instead of covalent attachment of biotin to residues bearing reactive amino groups, radioactive iodine atoms may be covalently attached to tyrosine residues in the polypeptide strand. In yet another embodiment, coumarin may be attached to residues bearing reactive carboxyl groups. The presence of coumarin may be detected optically or by immunochemical means. As will be plain, other combinations of label and signal may be employed.

Any of a large number of nucleic acid sequences may be employed in accord with this invention for use as probes in the detection of analytes. Included, for example, are target sequences in both RNA and DNA, as are the polynucleotide sequences that characterize various viral, viroid, fungal, parasitic or bacterial infections, genetic disorders

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or other sequences in analytes that it is desired to detect. Probes may be of synthetic, semi-synthetic or natural origin. Probe molecules include both polyribonucleotides and polydeoxynucleotides.

5           Although the presently preferred embodiment employs probes of approximately 30 base pairs, shorter probes may be used, provided they are capable of specifically and stably hybridizing to the target sequence. The probe may be designed to hybridize to  
10 either the sense or antisense strand of a DNA duplex. When the target is messenger RNA, however, the sequence of the probe should be complementary to it. The guanine and cytosine content of the probe is not critical for its ability to detect target sequences.

15           Synthesis of the labeled probe according to this invention is carried out in a series of steps. In the preferred embodiment, the probe molecule is activated at its 5' terminus by aminoalkylation. This may be achieved through the modification of the  
20 5' -OH group as its 5'-imidazol derivative using carbonyldiimidazole, which is subsequently displaced by an aminoalkyl compound such as hexamethylenediamine. A detailed description of a procedure for practicing this embodiment may be found in Wachter  
25 et al., "A Simple And Efficient Procedure For The Synthesis Of 5'-Aminoalkyl Oligodeoxynucleotides", Nucleic Acids Research, 14, pp. 7985-94 (1986). Other diamines can be used in place of the hexamethylenediamine of the preferred embodiment.  
30 Alternatively, the 5'-OH may be activated by other azolides [H. A. Staab, "Synthesis Using Heterocyclic Amides [Azolides]", Angew. Chemie International Edition, 1, pp. 351-67 (1962)], or with mestylene-sulfonyl or para-toluenesulfonyl groups, followed by  
35 amination by an  $SN_2$  displacement reaction.

          The label may be of any reasonable length. In order to permit covalent attachment of the linkage

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group and the label, the label preferably includes a reactive sulfhydryl group. Preferably, the label also includes reactive groups to which the bridging moiety or signal moiety may be attached. The label  
5 may be any organic molecule with the foregoing attributes. In the preferred embodiment, the label is a polypeptide and the bridging moieties are attached to the amino groups of ornithine or lysine residues and the carboxyl groups of aspartic acid  
10 and glutamic acid residues. Preferably, the polypeptide label has at least five bridging moieties attached to it. Alternatively, signalling moieties may be attached directly to these residues.

When the label is a polypeptide, the polypeptide strand may be synthesized chemically, relying  
15 on well known techniques. Alternatively, it may be derived from natural sources.

In order to minimize interference between bridging moieties and/or signalling moieties attached  
20 to a polypeptide strand, the residues to which the bridging or signalling moieties are attached should not be adjacent. Such spacing also facilitates access to the bridging moieties or reactive residues by second bridging moieties and signal moieties.

When the label is a polypeptide, the polypeptide strand includes a cysteine residue or other modified residue bearing a sulfhydryl group. In the preferred embodiment, the sulfhydryl group occurs at the amino or carboxyl terminus of the polypeptide  
25 strand. By this arrangement, the probe and label are non-adjacent, since the label is attached at the 5' terminus of the probe.

When the label comprises amino acid residues, the residues may be chosen from among those  
35 which are hydrophilic, such as glycine, asparagine, aspartic acid, glutamic acid, serine, threonine, histidine, arginine and tyrosine. One can also form



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a label which includes any of the natural amino acids or their D isomers provided that charge or steric hindrance between residues and between residues and bridging moieties, if present, do not interfere with access to the label by signalling moieties or second bridging moieties such as avidin or streptavidin.

Preferably, the label is attached to the activated probe by way of a linkage group formed by sequentially reacting an amino and sulfhydryl reactive, hetero bifunctional group with the activated probe and then with the label. Preferably, the linkage is formed by reacting the activated probe molecule with succinimidyl 4-[N-maleimidomethyl] cyclohexane-1-carboxylate resulting in release of N-hydroxy succinimide in the nucleophilic substitution reaction, followed by exposure of the derivatized probe to a label molecule bearing a reactive sulfhydryl group. Other compounds, such as meta-maleimidobenzoyl-N-hydrosuccinimide ester, N-succinimidyl [4-iodoacetyl] aminobenzoate, N-succinimidyl [4-bromoacetyl] and N-succinimidyl 3-[2-pyridylthio] propionate may be used to form a linkage group. The resulting label is held away from the probe by the linkage thus formed.

A useful feature of the labeled probes of this invention is that multiple signal moieties may be attached to each probe. Thus amplification of each primary recognition event is provided. In the preferred embodiment bridging moieties are attached to five lysine residues in the labeled polypeptide strand. Assuming that the signal produced is proportional to the number of label molecules present, this technique provides at least a five fold amplification of the signal. Further amplification is possible if a second bridging moiety, or combination of bridging moieties bearing multiple signalling moieties, is employed.

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Depending upon the label used, the signalling moiety may be chosen to bind directly to the label. Preferably, however, the signal is attached to a bridging moiety, and the bridging moiety binds to the label. For example, when biotin is used as a bridging moiety, avidin may be used as a second bridging moiety, and the avidin may have attached to it a fluorescein molecule as signalling moiety. Alternatively, a bridging moiety may be an antibody to the label. Yet another alternative within the scope of this invention would be to employ a first antibody as a bridging moiety, and a second antibody, bearing a signalling moiety, as a second bridging moiety.

In the preferred embodiment, biotin is used as a bridging moiety. Biotin is attached to the probe by incubating the labeled probe with biotin N-hydroxysuccinimide. This results in the covalent bonding of biotin molecules to the reactive amino groups present in the probe.

A modified version of this procedure may also be employed to label base moieties of nucleotides. Rather than attaching the linkage group of the present invention to a 5' -OH derivatized probe, the same linkage group may be used to attach a label to the reactive amino function of the modified base of Langer et al. (1981), and Ward et al., U.S. Patent 4,711,955, without significant changes in chemistry. In particular, since the linkage groups of the modified bases disclosed in the above documents bear reactive amine functions, they also will react with an amino and sulfhydryl reactive hetero bifunctional reagent, permitting attachment of a label thereto. When bases are labeled in this way, two linkage groups connect the label to the probe. The linkage group attached to the base by the method of Langer et al. may be termed a first linkage group, and the

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linkage group formed by reaction between the amine function of the modified base, and the amino and sulfhydryl reactive hetero bifunctional reagent and the label as a second linkage group. Bridging and signalling moieties may be attached to the labeled probe as previously described.

#### EXAMPLES

In order that this invention may be better understood, the following examples are set forth. These examples are for purposes of illustration only, and are not to be construed as limiting the scope of the invention in any manner.

#### Example 1

#### PREPARATION AND ACTIVATION OF A DNA PROBE

##### 15 a) Probe Synthesis

Four DNA probes were prepared using an Applied Biosystems Model 380A automated DNA synthesizer. The base sequences of the probes were:

1. 5' GAC CAC GCT GCT CTC CTG GGC ACA GCG GGA  
CGA ATC CGA
2. 5' CAC GCT GCT CTC CTG GGC ACA GCG GGA CGA
3. 5' TTG CTG GTA TAT CAT CTG CGT TTT TTC ATG
4. 5' TTG CTG GTA TAT CAC CTG CGT TTT TTC ATG

The synthesizer was programmed for DMTr<sup>a</sup>-off, using a manual ending method. The monomers used for constructing the probe were fully protected nucleoside-O-methylphosphoramidite monomers.

All of the above probes were anti-sense (complementary) to the gene coding for human tissue plasminogen activator ("tPA"). Probe 2 was a shorter

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<sup>a</sup> Dimethoxytrityl group.

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version of probe 1. Probe 3 and probes 1 and 2 were complementary to different regions of the tPA gene. Probe 4 was the same as probe 3 except at the underlined position, which was an intentionally introduced mismatch.

b) Aminoalkylation Of Probes

The 5' -OH group of the probe was activated as its 5' aminoalkyl derivative using a modified version of the method described by Wachter et al., 1986. During and after synthesis, the probes were attached to a solid support of aminopropyl controlled pore glass beads (Electronucleonics). After synthesis of the probe, the solid support, with the probe still attached to it, was taken up in a small reaction vessel having a medium porosity frit and a stop-cock. The probe was washed well under anhydrous conditions with dry acetonitrile. Carbonyldiimidazole (50 mg) was dissolved in 1 ml of anhydrous acetonitrile and this solution was introduced into the reaction vessel. The activation reaction was allowed to proceed for 30 minutes at room temperature with occasional shaking. After this time, the solid support was washed well with dry acetonitrile.

The 5'-aminoalkyl derivative of the probe was prepared by reacting the activated probe with a 1 ml solution of 0.3 M hexamethylenediamine dissolved in isopropanol. The nucleophilic substitution reaction was allowed to proceed for one hour at room temperature with occasional shaking. At the end of one hour, the reagents were washed off with isopropanol followed by methanol. The probe, while still attached to the solid support, was air dried.

After drying, the probe was treated with a 1 ml solution of thiophenol:triethylamine: dioxane (1:2:2) for 30 minutes to remove the phosphate protecting groups. The oligomer was cleaved from

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the solid support with concentrated ammonium hydroxide for one hour. The reaction mixture was then warmed to about 55° C for about 5 hours to remove the base protecting groups. The ammonium hydroxide was removed  
5 by drying the reaction mixture under vacuum.

After evaporation of the ammonium hydroxide, the activated probe was taken up in water and lyophilized. Initial purification, based on size, was performed using a 5 ml Sephadex G25 column (Pharmacia)  
10 equilibrated with 50 mM triethylammonium acetate at pH 7.0. Six 10 drop fractions were collected and the fraction containing the probe molecule was purified using either gel electrophoresis or high performance liquid chromatography ("HPLC").

When HPLC purification was employed,  
15 solvent A consisted of a mixture of 10% acetonitrile and 90% 50mM triethylammonium acetate ("TEAA") at pH 7.0, and solvent B was a mixture of 80% acetonitrile and 20% TEAA, also at pH 7.0.  
20 The solvents were applied to a C<sub>8</sub> column (Biorad) so as to create a gradient of 0% solvent B initially, increasing to 25% solvent B at 25 minutes. The solvent gradient was used to elute the oligomer from the column and the fraction containing the peak was  
25 collected and lyophilized.

When gel electrophoresis was used for purification, a 1.5 mm thick denaturing polyacrylamide gel was employed. The oligomer was visualized under short wave ultraviolet light and the appropriate  
30 band was cut out and eluted from the gel pieces using 100 mM triethylammonium bicarbonate at pH 7.0 for 4 hours at 60° C. The oligomer present in the eluant was then desalted on a 1 ml disposable C<sub>18</sub> column (Biorad) and was eluted from the column using 25%  
35 acetonitrile in water. The DNA content was determined by measuring absorbance at 2600 Å in a 1 ml diluted

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aliquot, and the purified derivatized probe lyophilized.

## Example 2

### LABELING ACTIVATED PROBES

#### 5           a) Polypeptide Synthesis

A 13 residue polypeptide was synthesized on an Applied Biosystems 430A automated peptide synthesizer according to the method of Merrifield [R. B. Merrifield, "Solid Phase Peptide Synthesis I: 10   Synthesis of a Tetrapeptide", J. Amer. Chem. Soc., 85, pp. 2143-54 (1963)]. The synthetic polypeptide had the sequence:

LysTyrGlyLysAsnSerLysProArgLysGluThrCys.

Following synthesis of the polypeptide, it was deprotected using anhydrous hydrogen fluoride in the 15   presence of the scavengers para-thiocresol, para-cresol and ethylmethyl sulfide. The scavengers were used in order to minimize the formation of side products. The reagents were employed in the following mixture:

10 ml 100% anhydrous hydrogen fluoride  
1 ml para-cresol  
1 ml ethylmethyl sulfide  
0.1 g para-thiocresol.

25           The crude peptide was purified by HPLC using a preparative C<sub>4</sub> or C<sub>18</sub> column using 0.1% trifluoroacetic acid in water as a solvent A and 0.1% trifluoroacetic acid in a mixture of 20% acetonitrile and 80% water as a solvent B. A gradient of 0% of 30   solvent B to 100% of the solvent B over 40 minutes was used to elute the peptide from the column. The purified peptide was detected by monitoring absorbance at 2200 Å. Aliquots containing the purified peptide were collected, lyophilized and stored at 4° C. The 35   composition of the polypeptide was confirmed by hydro-

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lyzing the polypeptide in 6N HCl for 24 hours at 110° C, followed by analysis of the hydrolysate on a Beckman amino acid analyzer.

b) Attachment Of Label To Probes

5            Approximately 1.5 A<sub>260</sub> units of the dried derivatized probe prepared according to Example 1 were suspended in 400 µl of 0.2M HEPES buffer at pH 7.7. To this was added 2 mg of succinimidyl 4-[N-maleimidomethyl] cyclohexane-1-carboxylate in 10   300 µl of acetonitrile. After mixing, the reaction was allowed to proceed for six hours at room temperature. The acetonitrile was then removed using a speed-vac. The aqueous solution, after pelleting the insoluble precipitate, was applied to a 5 ml G25 15   column equilibrated in 50 mM TEAA at pH 7.0, and the resulting condensate, 5'-maleimidomethyl cyclohexyl oligonucleotide, was eluted using the same buffer. Six 10 drop fractions were collected, and the fractions containing the reaction product purified using 20   a C<sub>8</sub> column under the conditions previously described for purification of the activated probe. The reaction product was lyophilized and its DNA content determined, after resuspending it in water, by measuring its absorbance at 2600 Å.

25            About 0.8 O.D. units (absorbance measured at 2600 Å of reaction product in 200 µl of HEPES buffer at pH 7.3 were mixed with 2 mg of polypeptide label. The reaction was allowed to proceed for one hour. The labeled oligonucleotide was purified using HPLC 30   under the conditions previously described for purification of the activated oligomer. The labeled probe eluted as a single peak having a lower retention time as compared to the unlabeled probe. The peak corresponding to the labeled probe was collected, 35   lyophilized and resuspended in 1 ml of water. Its

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absorbance at 2600 Å indicated that it consisted of 0.18 O.D. units.

c) Biotinylation Of Labeled Probes

The labeled probes from part b) of this Example were lyophilized in a speed-vac. The lyophilized DNA was suspended in 200 µl of 0.2 M HEPES buffer at pH 7.3. One mg of biotin-N-hydroxysuccinimide ester was mixed with 200 µl of acetonitrile. This mixture was added to a solution of labeled probe molecule, and the reaction was permitted to proceed for 4 hours at room temperature. The acetonitrile was evaporated using a speed-vac and the aqueous solution was purified by HPLC using a C<sub>8</sub> column under the conditions described in Example 1. The biotinylated labeled probe eluted from the column as a single peak well separated from the starting material. The biotinylated labeled probe was collected, lyophilized and suspended in 1 ml of a mixture of 10% acetonitrile and water. The mixture had an absorbance of 0.1 OD unit at 2600 Å.

Example 3

DETECTION OF DNA SEQUENCES USING THE LABELED PROBE

a) Preparation Of Dot Blots

Dot blots [F. C. Kafatos et al., "Determination Of Nucleic Acid Sequence Homologics And Relative Concentrations By A Dot Blot Hybridization Procedure", Nucleic Acids Research, 7, pp. 1541-22 (1979)] were prepared from a tPA containing plasmid, using serial dilutions of the plasmid DNA spotted on a nitrocellulose filter. Each dot also included 2 ng of sonicated salmon sperm DNA.



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b) Prehybridization Treatment Of Filters

Dried filters impregnated with analyte and salmon sperm DNA were wetted in water and in 6x SSC<sup>a</sup> and blotted dry. The filters were then treated with  
5 the following solution for 2-4 hours at 46° C.

- 5 ml 100% formamide
  - 2.5 ml 20x SSC
  - 0.5 ml 100x SSC
  - .25 ml 25 mM sodium phosphate solution (pH 6.5)
  - 10 0.75 ml water
- The above solution was used for treatment of 4 filters.

c) Hybridization

Hybridization of the labeled probe molecule to the test sample was carried out in a buffer solution with the following composition:  
15

- 4.5 ml formamide
- 2.5 ml 20x SSC
- 0.1 ml 100x Denhardt's<sup>b</sup> solution
- 0.2 ml 1M sodium phosphate at pH 6.5
- 20 0.2 mg/ml freshly boiled salmon sperm DNA
- 2.3 ml water

Labeled probe was used at a concentration of 50 ng/ml of hybridization solution. Hybridizations were performed using 5 ml of buffer solution in a sealed  
25 hybridization bag for two filters.

After hybridization, the filters were washed as follows:

- 6x SSC for 10 minutes at room temperature (2 changes)
- 3x SSC for 10 minutes at 46° C (2 changes)
- 30 1x SSC for 10 minutes at 55° C (2 changes)
- 0.1x SSC for 10 minutes at 55° C (2 changes)

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a 3.0 M sodium chloride  
0.3 M sodium citrate

35 b Denhardt et al., Biochem. Biophys. Res. Comm.,  
23, pp. 641-45 (1966).

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d) Filter Blocking

In order to minimize background, non-specific sites on the filters used for dot blots were blocked. Filters were washed in 0.1M Tris buffer at pH 7.5 for 5 minutes. The filters were then incubated in in 3% bovine serum albumin in a mixture of 0.1M Tris buffer and 0.45M sodium chloride at pH 7.5 for 30 minutes at 58° C, and blotted dry.

e) Detection

Bound probe was detected using the BluGene™ Non-Radioactive Nucleic Acid Detection System (BRL). Briefly, washed and blocked filters were incubated in freshly prepared streptavidin-alkaline phosphatase conjugate solution for 15 minutes at room temperature with gentle shaking. The conjugate solution was prepared by adding 10 microliters of 1 mg/ml stock solution to 10 ml of 0.1 ml Tris buffer at pH 7.5. Ten ml of this solution was used for two filters. Following this incubation, filters were washed two times for 15 minutes each with 200 ml of Tris buffer and 0.45 M sodium chloride at pH 7.5 for 15 minutes at room temperature.

For visualizing the hybridization of labeled probe molecules to sample DNA, the filters were incubated with a color development solution. The solution contained 44 µl of nitroblue tetrazolium in dimethylformamide (75 mg/ml in 70% dimethylformamide) and 33 microliters of 5-bromo-4-chloro-3-indoyl/phosphate (50 mg/ml in 100% dimethylformamide) to 10 ml 0.1 M Tris buffer containing 0.45 M sodium chloride and 50 mM magnesium chloride at pH 9.5. The incubation was carried out under low light for two to three hours in a sealed polypropylene bag. Color development was stopped by washing the filters in a solution consisting of 20 mM Tris buffer and 0.5 mM EDTA at

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pH 7.5. The filters were then blotted and dried under vacuum for two minutes prior to storage.

f) Results

When probe 1 was used for hybridization studies, 0.625 pg of target DNA could be detected visually when washes consisting of 2 changes of 6x SSC and 2 changes of 3x SSC all at 46° C for ten minutes were used. Similar results were obtained with probes consisting of shorter polynucleotides (probes 2 and 3), under similar washing conditions. None of the probes hybridized to the control DNA. In addition, probe 3, which had a GC content of 37%, produced negligible background when compared with probes 1 and 2 which had a higher GC content.

When probe 3 was used for hybridization studies and the filters were washed under high stringency conditions (i.e., the entire wash protocol set forth in Example 3) we could still visualize 10 pg of target DNA using colorimetric detection techniques.

When probe 4, which contains a single base mismatch, was tested under the same conditions, no probe DNA could be detected. Thus, our technique is sufficiently sensitive that it is capable of detecting alteration of a single base pair.

Example 4

DETECTION OF DNA SEQUENCES IN HUMAN GENOMIC DNA

It is also possible to use the labeled probes of this invention to detect the presence of particular nucleotide sequences in a sample of genomic DNA. In fact, as demonstrated in this example, use of the labeled probes of this invention in conjunction with the Southern blotting technique [E.M. Southern, "Detection of Specific Sequences Among DNA Fragments Separated By Gel Electrophoresis," J.Mol.Biol., 98,

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pp. 503-17 (1975)] permits detection of single copy genes (i.e., nucleotide sequences which occur only once in the genome of an organism) in a sample of genomic DNA.

5           Three probes were synthesized and labeled as described in Example 1. The probes had the following sequences:

1.   5' GAC CAC GCT GCT CTC CTG GGC ACA  
          GCG GGA CGA ATC CGA
- 10       5.   5' CTG GTA TAT CAT CTG CGT TTT TTC
6.   5' CTG GTA TAT CAC CTG CGT TTT TTC

Probes 1 and 5 are perfectly matched anti-sense probes to the human tPA gene and probe 6 is the same as probe 5 except at the underlined position, which is  
15 an intentionally introduced mismatch. Probe 1 of this Example is the same as probe 1 of Example 1. Probes 5 and 6 are complementary to a different region of the tPA gene.

a)   Preparation Of Human Genomic DNA

20           Human genomic DNA was isolated from cells of the human cell line GM 1416,48,4X, obtained from the Human Genetics Mutant Cell Repository in New Jersey, by the method of R. Cate, "Comparison Of The Methylation Patterns Of The Two Rat Insulin Genes",  
25 J. Biol. Chem., 258, pp. 6645-52 (1983). Aliquots of this DNA were cut with three different restriction enzymes to produce fragments of different lengths. The restriction enzymes were chosen so that they would not cut the DNA at positions within the target  
30 sequence.

For analysis with probe 1, 20 µg of human genomic DNA, prepared as above, was treated with 0.67 units of Bgl 2. To monitor digestions of genomic DNA, 10 µl of the reaction mixture was removed and  
35 added to 0.6 µg of DNA isolated from λ phage. Following treatment with the restriction enzyme, the